PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Eva KONTSEKOVA Peter FILIPCIK	Confirmation No.: 5434
Serial No.: 10/521,049	Examiner: Leavitt, Maria Gomez
Filed: November 1, 2005	Group Art Unit: 1633
For: TRANSGENIC ANIMAL EXPRESSING ALZHEIMER'S TAU PROTEIN	Atty. Dkt. No.: SONN:066US

THIRD FILIPCIK DECLARATION UNDER 37 C.F.R. § 1.132

I, PETER FILIPCIK, declare that:

1. I am a named inventor on the above-referenced patent application and I am an employee of Axon Neuroscience, the assignee of the above-referenced patent application. A copy of my Curriculum Vitae is attached.

2. It is my understanding that the Examiner in charge of this application has advanced an enablement rejection against claims 17-36. I am supplying this declaration to provide additional evidence of the enablement of the present claims.

3. This declaration provides evidence that we are able to produce transgenic animals with a predictable phenotype using gene constructs described in the present application. In our studies we have clearly shown that the phenotype induced by the transgenes, which are truncated tau, is robust, highly reproducible and predictable.

4. So far we have generated several independent transgenic lines (Tg line #318, Tg line #72 and Tg line #24) using DNA gene constructs encoding proteins, which have shown neurofibrillary (NF) pathology producing activity when expressed in brain cells.

5. Tg line #318 is described in the specification of the present patent application. The transgene construct used in the generation of transgenic rat line #318 encodes a truncated tau protein of amino acids 93-333 based on the numbering for the four-repeat containing tau 43 isoform. Amino acids 93-333 correspond to nucleotides 279-999 (SEQ ID NO: 3). The normal tau 43 protein is 383 amino acids. Thus, the truncated tau cDNA molecule used to generate rat line #318 is truncated at least 30 nucleotides uptsream of the stop codon of the full-length tau cDNA sequence coding for 4-repeat and 3-repeat tau protein; and the truncated tau cDNA molecule comprises SEQ ID NO: 9 (nucleotides 741-930). The same construct as used in the generation of trangenic rat line #318 also was used in the generation of transgenic rat line #72. The transgene construct used in the generation of transgenic rat line #24 encodes amino acids 93-302, which correspond to nucleotides 277-906 (SEQ ID NO: 12), based on the numbering used for isoform 44 (3-repeat tau). The normal tau 44 protein is 352 amino acids. Thus, the truncated tau cDNA molecule used to generate rat line #24 is truncated at least 30 nucleotides uptsream of the stop codon of the full-length tau cDNA sequence coding for 4-repeat and 3-repeat tau protein; and the truncated tau cDNA molecule comprises SEQ ID NO: 9 (nucleotides 741-930). The generation and studies of Tg line #24 were described in my previous declaration, which I signed on 12 September 2007.

6. The phenotype of these independent transgenic animals is very similar. The progress of sensory-motor impairment of animals from transgenic line #318 and transgenic line #24 is almost

identical. The onset and progression of neurodegeneration is the same in all three transgenic rat lines. The only difference we have observed is in the strength of the resulting phenotype when comparing Tg line #72 and Tg line #24. While behavioral features are almost the same, the life span of those animals containing 4 repeat tau (e.g. Tg line #72) is much shorter when compared to those animals containing 3 repeat tau region (e.g. Tg line #24) of human tau protein. However we are aware that the aggressiveness of neurodegeneration in human tauopathies including Alzheimer's disease may also be different in different patients.

7. Transgenic rat lines #318 and #24 exhibit neurofibrillary (NF) pathology. Transgenic rat line #24 developed neurofibrillary lesions in the brain stem, spinal cord, primary motor cortex, and hippocampus. Attached Figure 1 shows the staining of neurofibrillary lesions in the hippocampus and cortex of transgenic rat line #24 in the late stage of the disease.

8. Neurological examinations showed similar features in both the #24 and #318 transgenic rat lines. Sensory-motor impairment was measured by the "NeuroScale" method. NeuroScale represents a multi-test battery intended for the quantitative neurobehavioral evaluation of transgenic rats suffering from progressive sensorimotor neurodegeneration. Testing protocol enables complex sensorimotor, neuromuscular, and neurological assessment of rats at different age periods. Complex neurobehavioral characterization of rats involves basic observational assessment, examination of neurological functions and evaluation of rat neuromuscular functions by prehensile traction test, assessing forelimb muscle strength and assessment of sensorimotor coordination abilities using a beam walking test. This experimental strategy can reveal impairment, which could otherwise be hidden, and permits observation of changes caused by chronic neurodegenerative process. As shown in attached Figure 2, the progress of sensory-

motor impairment of animals from transgenic line #318 and transgenic line #24 was almost identical. The onset and progression of neurodegeneration was the same in both transgenic rat lines. The transgene was transmitted to subsequent offspring generations and the phenotype remained unchanged even in the 4th generation of offspring.

9. Another measure of cognitive impairment is the object recognition test (ORT). ORT is used to measure object recognition memory, which is the ability to discriminate between objects that have been previously encountered and those that have not been. A spontaneous exploratory activity can be used for measurement of memory function in rats. ORT in animals is based on the natural preference of investigating a novel object rather than a familiar object. The intensity of memory storage can be tested using various types of delays between the first (presentation) and second (challenge) trial, in which the new object replaces a familiar object. As shown in attached Figure 3, transgenic rats from line #24 suffer from early cognitive impairment in the object recognition test.

10. According to our latest data we have concluded that the final neurofibrillary tangle (NFT) load in the terminal stage of life of transgenic animal lines, which were produced using different truncated tau gene constructs, is independent of human tau expression levels as shown in Figure 4. We have quantified those mAb AT8-immunoreactive tangle bearing neurons that display characteristic fibrillary structures in the neuronal cytoplasma. (A) Representative pathological structures present in the reticular formation of the brain stems of transgenic rats stained by mAb AT8 are depicted: perinuclear tangles, intracellular tangles that fill the neuronal soma and neurofibrillary tangles distributed in the somatodendritic compartment (Scale bar = 10 μm). (B) Total number of neurons and neurofibrillary tangles were determined in male rats from the

SHR72 (7.5 months old) and SHR318 (10.5 months old) transgenic lines. (C) The final NFT loads in SHR72 and SHR318 male rats showed no significant difference ($P = 0.71$). Bars represent mean values for each group ± SEM.

11. Figure 5 shows that truncated tau transgenic expression in two different lines does not cause neuronal loss in the brainstem and hippocampus of transgenic animals. (A) Stereological analysis of neuronal loss in GRN in 7.5-month old transgenic SHR72 males and 10.5-month-old SHR318 males did not reveal any difference in total neuron numbers in comparison with age-matched wild-type rats (t-test: SHR72 vs wt, $P > 0.05$; SHR318 vs wt, $P > 0.05$). (B) Age related neuronal loss was present in GRN in the SHR rat strain. 7.5-month-old wild type SHR rat males display on average 25.4% fewer Nissl-stained neurons (Bonferroni's post hoc test, $P<0.01$) and 10.5-month-old animals on average 39.7% fewer Nissl-stained neurons (Bonferroni's post hoc test, $P<0.001$) than 5-month-old animals. Bars represent mean values for each group ± SEM. (C) Cresyl violet staining of hippocampal pyramidal neurons (CA 1 area) in the transgenic and control rats did not show any visible differences (Scale bar = 50 μm). (D) Stereological analysis of the total number of pyramidal cells in the hippocampal area CA 1-3 revealed no statistically significant differences between transgenic and wild type rat males in either of the investigated groups (t-test: SHR72 vs wt, $P > 0.05$; SHR318 vs wt, $P > 0.05$).

12. Another striking feature of animals produced by transgenic truncated tau expression is that the observed phenotype is not dependent on genetic background. After the transfer of the transgene from the genetic background of the hypertensive SHR strain into the normotensive Wistar strain (WKY) we have observed, in this new genetic environment, almost the identical phenotype at the level of biochemical examination as well as in behavioral measurements.

Although we expected less aggressive neurodegeneration in WKY animals, this was not the case. To illustrate this phenomenon see data included in Figure 6, showing that neurobehavioral impairment of the WKY transgene #72 goes in parallel with that of SHR transgene #72 as demonstrated by an almost identical increase in the neuroscale score. Figure 7 shows no significant influence of the strain differences on phospho-tau level in CSF at the biochemical level.

13. The resulting phenotype was synergistic in those animals that we have generated by crossing animals encompassing human truncated tau with 4 and 3 repeat. As shown in Figure 8, sensorimotor functions measured by beam walking test were significantly more impaired in transgenic line SHR24/72 (expressing both 3R and 4R truncated tau proteins) when compared with transgenic lines SHR24 (expressing 3R truncated tau) and SHR72 (expressing 4R truncated tau). To further demonstrate the synergistic effect of 3R and 4R truncated tau we performed neuroscale evaluation and we found that the complete neurobehavioral phenotype was significantly more impaired in transgenic line SHR24/72 (expressing both 3R and 4R truncated tau proteins) when compared with transgenic lines SHR24 (expressing 3R truncated tau) and SHR72 (expressing 4R truncated tau) as shown in Figure 9.

14. Neurofibrillary pathology is the most important and earliest immunohistochemical finding in Alzheimer's disease. Thus, an animal model that exhibits neurofibrillary pathology is a useful model of Alzheimer's disease. Among the published animal models our transgenic model belongs to those with the most aggressive neurodegenerative phenotype moreover resembling fundamental neuropathological features of brain typical for Alzheimer's disease sufferers.

Furthermore, all the transgenic lines we have generated exert stable phenotype even after several years of continual breeding.

15. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 20.11.2008

Peter Filipcik

PETER FILIPCIK

Figure 1

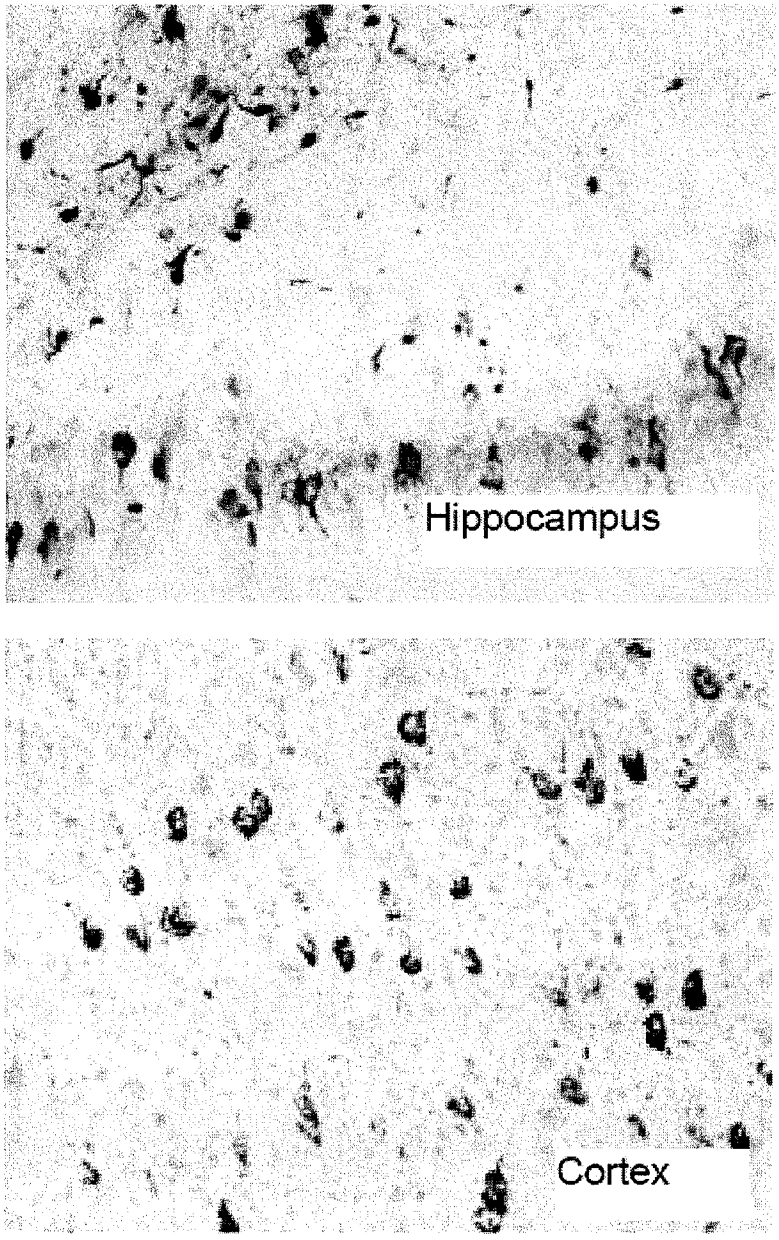


Figure 2

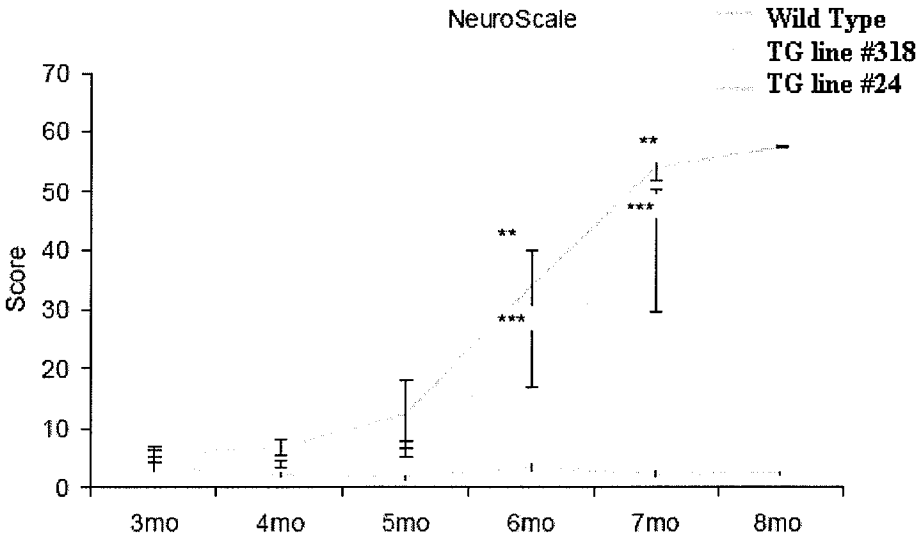

NeuroScale
Wild Type
TG line #318
TG line #24
Score
70
60
50
40
30
20
10
0
3mo
4mo
5mo
6mo
7mo
8mo
**
***
**
***

Figure 3

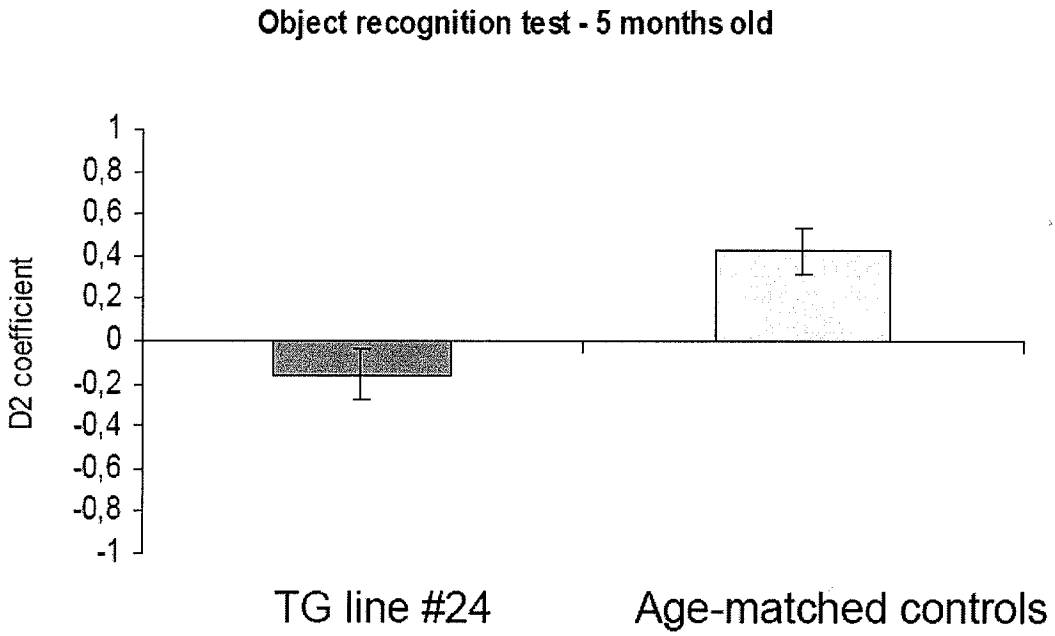

Object recognition test - 5 months old
D2 coefficient
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1
TG line #24
Age-matched controls

Fig.4

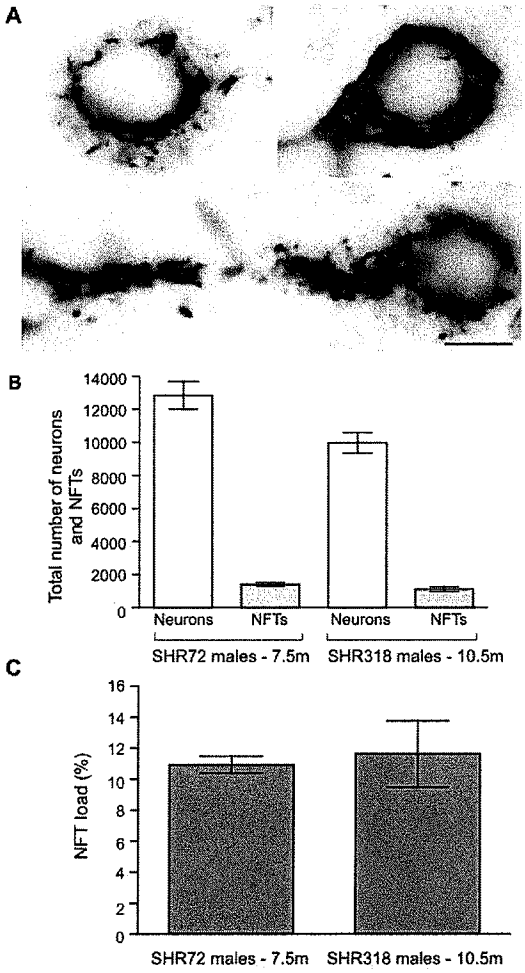

A
B
Total number of neurons and NFTs
14000
12000
10000
8000
6000
4000
2000
0
Neurons
NFTs
Neurons
NFTs
SHR72 males - 7.5m
SHR318 males - 10.5m
C
NFT load (%)
16
14
12
10
8
6
4
2
0
SHR72 males - 7.5m
SHR318 males - 10.5m

Fig. 5

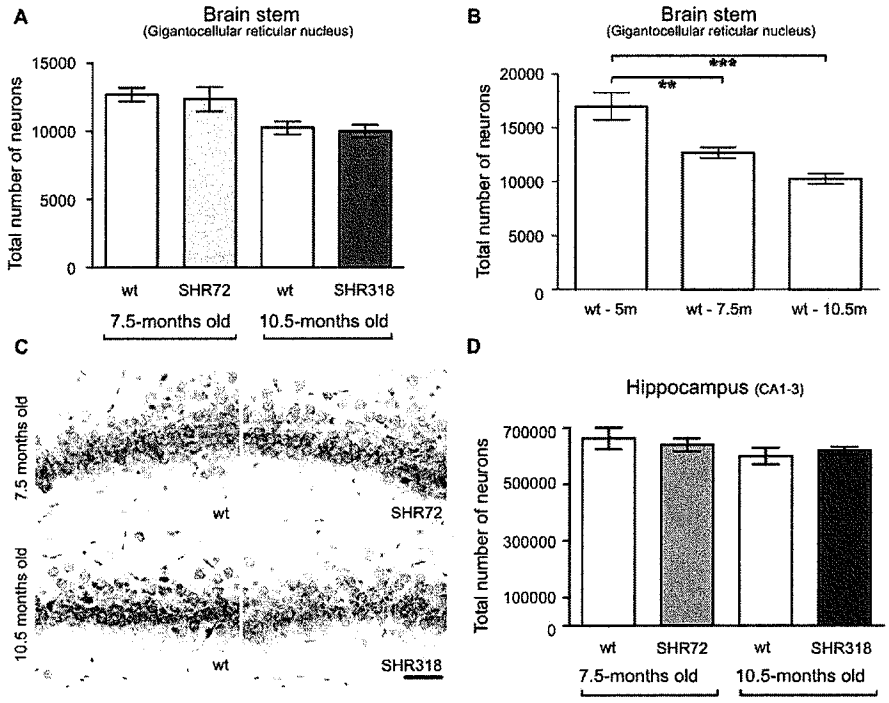

A
Brain stem
(Gigantocellular reticular nucleus)
Total number of neurons
15000
10000
5000
0
wt
SHR72
wt
SHR318
7.5-months old
10.5-months old
B
Brain stem
(Gigantocellular reticular nucleus)
Total number of neurons
20000
15000
10000
5000
0
**
***
wt - 5m
wt - 7.5m
wt - 10.5m
C
7.5 months old
10.5 months old
wt
SHR72
wt
SHR318
D
Hippocampus (CA1-3)
Total number of neurons
700000
500000
300000
100000
0
wt
SHR72
wt
SHR318
7.5-months old
10.5-months old

Fig. 6

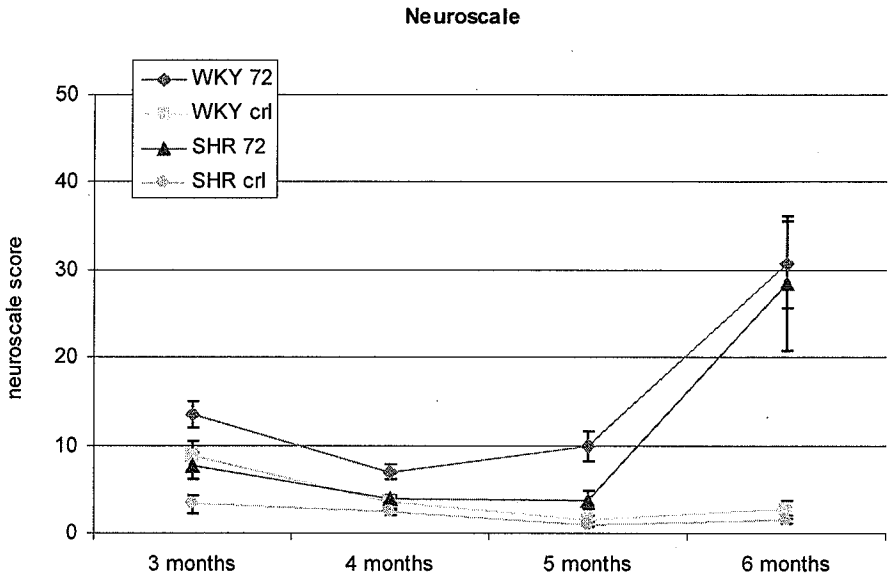

Neuroscale
WKY 72
WKY crl
SHR 72
SHR crl
neuroscale score
50
40
30
20
10
0
3 months
4 months
5 months
6 months

Fig. 7

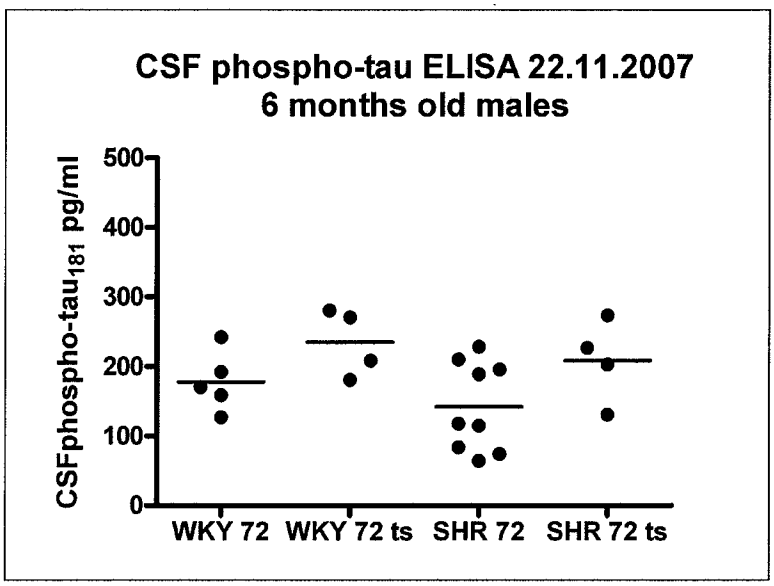
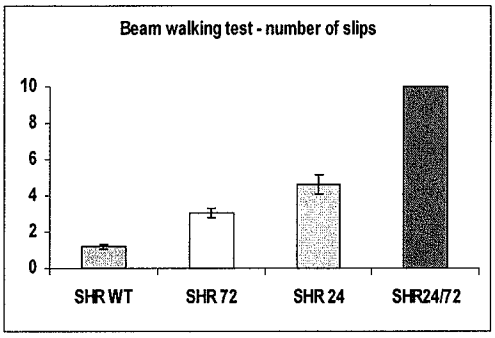

CSF phospho-tau ELISA 22.11.2007
6 months old males
CSFphospho-tau181 pg/ml
500
400
300
200
100
0
WKY 72
WKY 72 ts
SHR 72
SHR 72 ts

Fig. 8.


Beam walking test - number of slips
10
8
6
4
2
0
SHR WT
SHR 72
SHR 24
SHR24/72

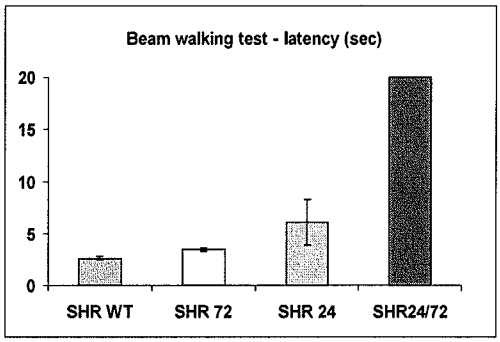
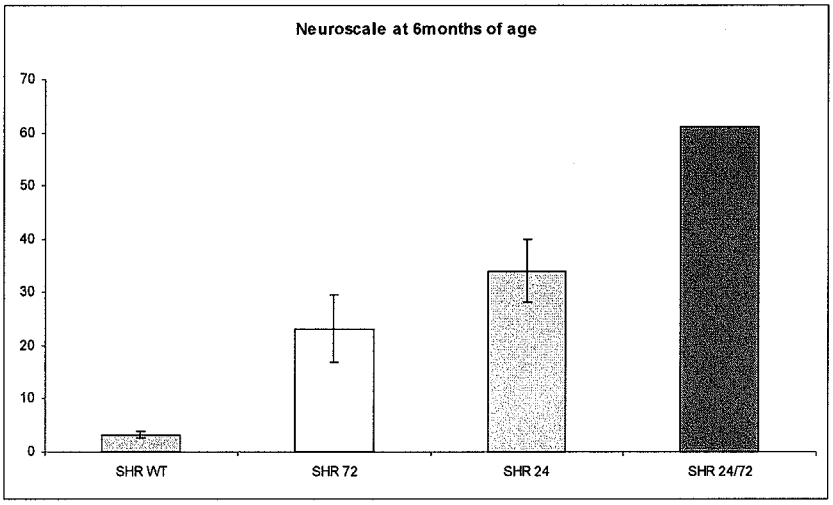

Beam walking test - latency (sec)
20
15
10
5
0
SHR WT
SHR 72
SHR 24
SHR24/72

Fig. 9.


Neuroscale at 6months of age
70
60
50
40
30
20
10
0
SHR WT
SHR 72
SHR 24
SHR 24/72